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SIMPLE TWO-STEP SEDIMENT EXTRACTION PROCEDURE FOR USE IN GENOTOXICITY AND TOXICITY BIOASSAYS

by

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ABSTRACT

Three simple sediment extraction procedures, using Milli Q water, DMSO and methanol, were applied to 16 sediment samples collected from the Thames River, Ontario, Canada. The three extracts were tested for the presence of toxicity and genotoxicity using the Microtox, Toxi-Chromotest and Mutatox (with and without S-9) procedures. Results of the study indicated that the Mutatox, genotoxicity screening test was an extremely sensitive procedure responding to chemicals in all three types of extracts. Methanol was found to be more efficient than DMSO in extracting toxic and genotoxic chemicals from these sediments. Procedural details and results are discussed.

RÉSUMÉ

Trois méthodes simples d'extraction pratiquées sur des sédiments, avec de l'eau Milli Q, avec du diméthylsulfoxyde ainsi que du méthanol, ont été appliquées à l'analyse de 16 échantillons de sédiments prélevés dans la rivière Thames en Ontario, Canada. Les chercheurs voulaient déceler la preuve de toxicité et de génotoxicité dans les trois extraits au moyen des modes opératoires du Microtox, du Toxi-Chromotest et du Mutatox (avec et sans S-9). Les résultats ont montré que la méthode Mutatox, un essai de dépistage de la génotoxicité, était une méthode excessivement sensible qui donnait des résultats avec les trois types d'extraits. Il a été constaté que le méthanol se révélait plus efficace que le diméthylsulfoxyde pour l'extraction des agents chimiques toxiques et génotoxiques contenus dans les sédiments. Les méthodologies sont expliquées et les résultats sont analysés.

MANAGEMENT PERSPECTIVE

Procedures for the extraction of sediments for toxicity and genotoxicity studies are becoming increasingly complicated, time consuming and expensive. The original concept of using short-term bioassays was, to quickly and efficiently test water, particulates and sediment samples for toxicant/genotoxicant responses so that samples could be prioritized for the more costly chemical analyses.

In this study we have evaluated three extremely simple, quick and inexpensive sediment extracting procedures. The extracts, when tested by three tests (Microtox, Mutatox and Toxi-Chromotest) from our battery of tests approach, indicated that these extraction procedures showed great potential and should be part of future research projects.

The Mutatox test was also shown to be one of the more sensitive and reliable genotoxic and toxicity screening tests and warrants inclusion into all "battery of tests" approaches.

PERSPECTIVE - GESTION

Les méthodes d'extraction pratiquées sur les sédiments en vue d'une analyse de la toxicité et de la génotoxicité se compliquent, prennent de plus en plus de temps et sont aussi de plus en plus coûteuses. À l'origine, on a voulu mettre au point des essais biologiques rapides afin de mesurer avec efficacité et rapidité la toxicité et la génotoxicité d'échantillons d'eau, de particules et de sédiments de façon qu'il soit possible de classer les échantillons par ordre d'importance avant de procéder à des analyses chimiques qui sont plus coûteuses.

Au cours de la présence étude, nous avons évalué trois méthodes extrêmement simples, rapides et peu coûteuses d'extraction sur des sédiments. Les fractions extraites qui ont été soumises à trois essais (Microtox, Mutatox et Toxi-Chromotest) de notre batterie d'essais, ont montré que les méthodes d'extraction essayées étaient prometteuses et qu'elles devaient être éventuellement intégrées à des projets de recherche.

Il a été établi que l'essai Mutatox constitue l'un des tests de dépistage de la toxicité et de la génotoxicité parmi les plus fiables et les plus sensibles et qu'il mérite d'être inclus dans toutes nos "batteries d'essais".

INTRODUCTION

Either directly or indirectly sediments are usually the final repository for the multitude of known and unknown chemicals entering the aquatic ecosystem. Many studies have shown that sediment extracts obtained by a variety of extraction procedures react with various short term bioassays to produce toxic or genotoxic effects (Trevors, 1984; Dutka, 1988; Samoiloff, 1988; Henebry and Ross, 1989). Also the application of these short-term bioassays to environmental samples has revealed that there is no single screening test which is responsive to all conditions. This realization has led to the concept of using a battery of tests to ascertain the ecological impacts of effluents, discharges and contaminated sediments. However, there is no standardized protocol for extraction of sediments in order to test for their contained toxicant/genotoxicant activities.

A variety of extraction techniques have been used with varying degrees of success (Dutka et al., 1981; Ross et al., 1987; Swartz, 1987; Burton, 1989, and Parkhurst et al., 1989), and one never knows how much of the bound organic/inorganic material has been solubilized or transformed with each extraction process.

One of the goals of the "battery of tests" approach has been to quickly, efficiently and inexpensively screen a variety of waters, suspended particulates and sediments and prioritize them for more intensive and costly chemical analyses. If the extraction procedures are too time consuming and costly then the battery of test option for screening the samples is negated.

In this study we have evaluated the use of two very simple organic extraction procedures on 16 sediments. The sensitivity and specificity of the solvents DMSO and methanol for extracting toxic or genotoxic bioassays from the "battery of tests" approach (Dutka, 1988) and comparing the results to those obtained from our routine first-step sediment extraction procedure using Milli-Q water (Dutka and Kwan, 1988). Results of this study are presented and discussed.

METHODS

Samples

Sediments were collected from 5 sites in triplicate (15 metres apart) from the Thames River in and on the outskirts of London, Ontario, Canada. A sixth single site situated well downstream of the urban influence (25 km) was also collected to evaluate particulate and toxicant transport potential by the Thames River. Sediment samples were only collected from the top 2-3 cm surface layer, mixed and placed into appropriate containers, refrigerated and returned to the laboratory.

Sediment Extraction

In the laboratory, each sediment sample was thoroughly homogenized and three 100 gram aliquots were removed. Each sediment aliquot

was mixed with one extracting solvent, either DMSO 100%, methanol 100% or Milli Q water in a 1:1 (wt. weight:volume) ratio in an acid washed B.O.D. bottle and shaken vigorously for two minutes.

Each of the mixtures was then centrifuged at 10,000 rpm for 20 min in a refrigerated centrifuge. The supernatants were used in the toxicity and genotoxicity screening tests.

Extract Concentrations Tested

Prior to conducting any bioassays, the maximum allowable concentration (MAC) of the organic solvents had to be established. MAC is defined as the concentration (%) of solvents which does not produce an effect on the test organisms. The MAC of Dimethyl Sulfoxide (DMSO) for the Microtox test was 0.5% and for the Toxi-Chromotest was 2.0%. For methanol the MAC with the Microtox test was 4.0%, and with the Toxi-Chromotest it was 6.0%. In the Mutatox procedure the MAC of DMSO and methanol was 3.8%.

Toxicity Assessment

Three screening assays, two for acute toxicity (Microtox and Toxi-Chromotest) and one for chronic toxicity (Mutatox) were used. The Microtox test was performed using the luminescent bacterium Photobacterium phosphoreum and the procedure detailed in the Beckman

Microtox Operation Manual (1982) with a 15 min. contact time (Dutka and Kwan, 1982). The Toxi-Chromotest (Orgenics Ltd., Box 360, Yavae, Israel) which is a relatively new bacterial colorimetric assay based on the ability of toxicants to inhibit the de novo synthesis of an inducible enzyme, beta-galactosidase, in an E. coli mutant, was also used to test the sediment extracts.

A new unproven environmental genotoxic screening test, the Mutatox test, was evaluated in this study. The Mutatox test is based on the use of a dark mutant strain M169 of Photobacterium phosphorium and this test will pick up chemicals which are (a) DNA damaging agents, (b) DNA intercalating agents, (c) direct mutagens which either cause base substitution are frame shift agents, and (d) DNA synthesis inhibitors (Microbics Corporation, Carlsbad, California). This is a simple 18 hr test performed at 22±2°C for which sample sterilization is not necessary. Toxicity of the test sample can be determined simultaneously with its genotoxicity (Ulitzur, 1986). The Mutatox test can be performed with or without the addition of the S-9 liver homogenate fraction. Details of the procedure are as follows: (1) prepare 14 12 x 50mm test cuvettes, (2) add 1.0 mL assay mixture into cuvette #1 (assay mixture = media + culture in 100:1 ratio), (3) add .5 mL assay mixture into cuvettes 2-14, (4) add .02mL sample into cuvette #1, (5) dilute sample in cuvette #1 into cuvettes 2-10 (1:2 serial dilutions) (6) incubate cuvettes at 20 - 24°C and (7) read light level for each cuvette at 18± hr.

In this study genotoxic activity is defined as when the test sample (a) without S-9 addition produces four times the luminescence level produced by the control (Milli Q water) and (b) with S-9 addition, produces two times the luminescence level produced by the control. A toxic effect is noted when the maximum luminescence level produced by the tested sample, as measured by the Microtox photometer, is below the maximum luminescence level produced in the control sample. Toxic activity can also be observed visually (microscope) by comparing the bacterial density of the control sample with the test sample.

Studies by Ulitzur (1986) have shown that the Mutatox test using the dark mutant of Photobacterium phosphoreum is able in many instances to detect lower minimal genotoxic concentrations than the Ames Test. Numerous studies with pure chemicals comparing the Mutatox procedure with the Ames test have been performed (Microbics Corp.) and some of the results are shown in Table 1.

RESULTS AND DISCUSSION

Tables 2, 3 and 4 present the data obtained from the three bioassays used to screen the 16 sediment extracts.

In Table 2, water extracted sediments, it can be seen that the Toxi-Chromotest and the Mutatox toxicity screening tests were negative in all 16 samples from the six sites. In contrast, the Mutatox test, with and without the addition of S-9, indicated the presence of geno-

toxic agents in all 16 samples. In all instances, the samples with S-9 were less responsive than the samples tested without the S-9. This may be a real effect and the S-9 addition dampens the amount of luminescence produced. The Microtox test was found to produce a positive response to the toxicants in 9 of the samples (Sites 1, 2 and 3).

In Tables 3 and 4, it can be seen that the Microtox test indicated that 9 and 12 samples out of the 16 contained toxicants and with the sole exception of sample 4a extracted with methanol, DMSO and methanol extracted sediments generally produced lower values than those found in the Milli Q water extracted sediments (Table 2). From the Microtox test results shown in Tables 2, 3 and 4, it would appear that methanol is a slightly better solvent than DMSO and Milli Q water. However, samples 1a and 1b appear to contain toxicants some or all of which are water soluble, and if any solely organic soluble toxicants were present, they are in too low of a concentration to be picked up in the much greater diluted methanol and DMSO extracts. In the Microtox test the first water extracted sample tested would contain 90.8% of the original 1:1 extract while the DMSO sample would contain 0.91% and the methanol sample would contain 1.82%. Thus in the DMSO sample the Microtox test is responding to ≈1/100, the sample concentration and in the methanol sample to ≈1/50 sample concentration. It is surmised that methanol as an organic solvent is slightly more efficient than DMSO in these tests due to methanol's lesser toxicity to the Photobacterium phosphoreum thus allowing for a greater concentration of the sample to be reacted with the test organism.

However, notwithstanding the above, as shown in sample 2b, Tables 3 and 4 and possibly samples 4a, b and c, solvent specificity for contaminants may also have a role.

In Table 2, Microtox tests, seven samples, 4a to 6a are reported as showing light stimulation. This responses is believed to be due to hormesis, which is defined as stimulating effects caused by low levels of potentially toxic agents (Stebbing, 1982). There is confirmation of this belief in that samples 4a to 5c are positive for toxicant activity with the DMSO and methanol extracts and 6a is positive in the Mutatox and Toxi-Chromotest (Tables 3 and 4).

The Toxi-Chromotest was less sensitive to the toxicants present in the sediment extract than the Microtox or Mutatox tests. In Table 2 it can be seen that no samples were positive in the 16 Milli Q water extracted sediments while with the DMSO extracts 4 samples were toxic, 4c, 5a, 5b and 5c, while 11 methanol extract samples were positive, 2c to 6a inclusive. Even though the bacterial strain used in the Toxi-Chromotest has been shown to be sensitive to a wide spectrum of toxic chemicals (Reinhartz et al., 1985) it is apparent that the concentrations of water soluble compounds were not high enough to be detected.

Again, similar to the Microtox test results, Tables 3 and 4 demonstrate that direct methanol extraction is the procedure which produces the greater number of positive results indicating toxicant presence. It is not entirely clear whether solvent specificity or toxicant load or both play a role in these observed results, neverthe-

less it would appear that the Toxi-Chromotest responds to different water soluble compounds than either the Microtox and Mutatox tests and these compounds are not present or too dilute for this test to respond to.

The Mutatox test for genotoxicity without S-9 addition was positive in all Milli-Q water extracted sediments, and in 15 of the 16 DMSO and methanol extracted sediments (Tables 2, 3 and 4). The DMSO positive results appear to be slightly higher than the methanol extract results even though the methanol extracts contained approximately 2X more of the original sample.

The addition of S-9 to the Mutatox test resulted in all of the Milli Q water extracts showing positive for genotoxicant presence, although to a lesser degree than that observed without S-9 addition. However, results from organic solvent extracted sediments indicated that only two samples, 2c and 3a, were positive in the DMSO extracts and six samples, 1c to 3b inclusive were positive with the methanol extracts. All of the results with the exception of 3a sample (methanol extract) were all borderline positives.

With all of the sediments, the Milli Q water extracting procedure was the much more selective procedure in extracting genotoxic chemicals for testing by the Mutatox procedure, and as noted earlier the addition of S-9 tends to dampen or dilute the genotoxicity response. Of the two solvent extracting procedures, methanol appears to be more selective, although it is believed this may be solely due to the greater toxicant load carried by the less diluted methanol extract.

All Milli Q water samples were negative for toxicants as measured by the Mutatox test, with and without S-9 addition. The methanol extracts without S-9 addition indicated that 15 of the 16 samples contained toxicants while only three DMSO extracts indicated the presence of toxicants. When S-9 was added to the system, all DMSO extracts were negative for toxicity and only 3 of the methanol extracts were positive for toxicants. The addition of S-9 mix to any of the extracts, Milli Q, DMSO or methanol tends to decrease the sensitivity of the test and appears to have no redeeming features.

In summation the direct extraction of sediments by Milli Q water appears to be a simple, inexpensive and effective means of extracting toxicants and genotoxicants for testing via the Microtox and Mutatox procedures. Methanol, possibly due to its lesser toxicity to the organisms used in the various bacterial based screening tests, proved in this study to be more efficient than DMSO in extracting toxicants and genotoxicants.

The Mutatox test has proven to be a very sensitive procedure, with both Milli Q water and solvent extracted sediments, for showing the presence of genotoxicants and toxicants. The results observed with S-9 addition were similar to those observed in earlier studies and therefore for general screening procedures we would not recommend that S-9 be used. Similarly the Toxi-Chromotest with its lack of specificity and sensitivity would not be recommended as a test to be included in the battery of tests approach, while the Mutatox test should be a preferred member.

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Table 1. List of chemicals tested for comparison with the Ames test.

Chemicals	TEST		
	Ames	Mutatox	Carcinogenicity
Dioxane	-	-	+
Ethyl Methanesulfate	+	+	+
8-OH Quinoline	+	+	-
Mannitol	-	-	-
Menthol	-	-	-
NaN3	+	-	?
Orange G	-	-	-
Phenol	-	+	-
Reserpine	-	-	+
Salicylic Acid	-	-	-
Safrole	-	+	+
Novobiocin	-	+	-
4-Nitroquinoline-1-Oxide	+	+	+
NTG	+	+	+
Proflavine	+	+	?
Propyl Gallate	-	+	+/-
Quinacrine	+	+	+
Acetamide	-	-	+
2-Aminobiphenyl	-	+	+
1,2 Benzanthracene	+	+	+
Caprolactam	-	-	-
Cyclophosphamide	+	-	+
Chloramphenicol	-	-	?
2-Aminofluorene	+	+	+
9-Aminoacridine	+	+	?
Acetylaminofluorene	+	+	+
Acridine Orange	+	+	?
2-Anthramine	+	+	+
Benzidine	+	+	+
Benzo(a)pyrene	+	+	+
Benzoin	+/-	+	-
Caffeine	-	+	?
Eugenol	-	+	+
Mitomycin C	+	+	+
Nalidixic Acid	-	+	

Data obtained from Microbics Corporation

Table 2. Toxicity and genotoxicity data from three bioassay using Milli Q reagent grade water as extracting solvent.

Sample #	Microtox % Sample Producing (EC_{50})	Toxi-Chromotest (% Inhibition)	Mutatox Toxicity -(S-9)	Mutatox Toxicity +(S-9)	Mutatox Genotoxicity XTimes Control Light Production -(S-9)	Mutatox Genotoxicity XTimes Control Light Production +(S-9)
1a	27.6%	ND[2]	ND	ND	8.1X	3.5X
b	18.5%	ND	ND	ND	10.0X	4.7X
c	11.3%	ND	ND	ND	13.1X	7.7X
2a	17.4%	ND	ND	ND	11.3X	4.3X
b	32.0%	ND	ND	ND	11.3X	3.7X
c	17.7%	ND	ND	ND	11.3X	5.7X
3a	30.3%	ND	ND	ND	10.6X	4.7X
b	15.3%	ND	ND	ND	10.6X	3.7X
c	35.8%	ND	ND	ND	8.1X	3.6X
4a	STIM[1]	ND	ND	ND	11.3X	3.9X
b	STIM	ND	ND	ND	11.3X	4.9X
c	STIM	ND	ND	ND	15.0X	5.9X
5a	STIM	ND	ND	ND	11.9X	5.9X
b	STIM	ND	ND	ND	11.9X	5.1X
c	STIM	ND	ND	ND	15.0X	10.0X
6a	STIM	ND	ND	ND	7.5X	4.9X

STIM[1] - Stimulated
ND[2] - Not Detected

Table 3. Toxicity and genotoxicity data from three bioassay using 100% Dimethyl Sulfoxide (DMSO) as extracting solvent.

Sample #	Microtox % Sample Producing (EC_{50})	Toxi-Chromotest (% Inhibition)	Mutatox Toxicity -(S-9)	Mutatox Toxicity +(S-9)	Mutatox Genotoxicity XTimes Control Light Production -(S-9)	Mutatox Genotoxicity XTimes Control Light Production +(S-9)
1a	ND[1]	ND	ND	ND	9.8X	ND
b	ND	ND	ND	ND	10.9X	ND
c	38.8%	ND	ND	ND	11.1X	ND
2a	33.8%	ND	ND	ND	10.7X	ND
b	36.1%	ND	ND	ND	8.9X	ND
c	40.8%	ND	ND	ND	10.4X	2.3X
3a	39.5%	ND	ND	ND	11.8X	2.0X
b	ND	ND	ND	ND	11.8X	ND
c	47.1%	ND	ND	ND	6.0X	ND
4a	ND	ND	ND	ND	6.7X	ND
b	ND	ND	ND	ND	6.7X	ND
c	ND	3.3%	ND	ND	6.9X	ND
5a	34.9%	3.8%	ND	ND	8.2X	ND
b	30.0%	12.7%	T[2]	ND	8.4X	ND
c	31.4%	4.6%	T	ND	8.0X	ND
6a	ND	ND	T	ND	ND	ND

ND[1] - Not Detected
T[2] - Toxic

Table 4. Toxicity and genotoxicity data of three bioassay using 100% Methanol as extracting solvent.

Sample #	Microtox % Sample Producing (EC_{50})	Toxi-Chromotest (% Inhibition)	Mutatox Toxicity -(S-9)	Mutatox Toxicity +(S-9)	Mutatox Genotoxicity XTimes Control Light Production -(S-9)	Mutatox Genotoxicity XTimes Control Light Production +(S-9)
1a	ND[1]	ND	T[2]	T	4.0X	ND
b	ND	ND	ND	ND	4.3X	ND
c	44.2%	ND	T	ND	4.3X	2.0X
2a	46.0%	ND	T	ND	4.3X	2.0X
b	ND	ND	T	ND	4.7X	2.3X
c	13.3%	9.9%	T	ND	ND	2.0X
3a	11.9%	8.0%	T	ND	4.7X	3.0X
b	32.7%	7.7%	T	ND	4.0X	2.4X
c	25.4%	13.9%	T	T	6.7X	ND
4a	9.0%	29.7%	T	T	8.0X	ND
b	27.0%	13.1%	T	ND	7.0X	ND
c	28.2%	11.4%	T	ND	7.3X	ND
5a	19.7%	8.8%	T	ND	8.3X	ND
b	20.9%	7.2%	T	ND	8.3X	ND
c	24.0%	10.0%	T	ND	8.7X	ND
6a	ND	10.1%	T	ND	8.0Xx	ND

ND[1] - Not Detected
T[2] - Toxic